(54) Title: MODIFIED INTERLEUKIN-1 INHIBITORS

(57) Abstract

DNA molecules which encode improved biologically active Interleukin-1 inhibitors are disclosed. DNA molecules that encode improved Interleukin-1 inhibitors incorporated into expression vectors are also disclosed. In addition, host cells transformed with expression vectors that contain DNA molecules encoding improved IL-1 inhibitors and a method of producing improved biologically active IL-1 inhibitors are disclosed. Essentially pure, improved, biologically active IL-1 inhibitors are disclosed.

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MODIFIED INTERLEUKIN-1 INHIBITORS

FIELD OF THE INVENTION

The present invention relates to novel compounds which inhibit the activity of Interleukin-1   (IL-1).

5   BACKGROUND OF THE INVENTION

Interleukin-1   (IL-1) is a polypeptide that is produced after infection, injury, or antigenic challenge.   The macrophage is a primary source of IL-1; epidermal, epithelial, lymphoid, and vascular tissues also synthesize IL-1. When IL-1 circulates, it acts like a hormone and induces a broad spectrum of systemic changes

10   in neurological, metabolic, hematologic, and endochronologic systems.   Some of the IL-1 that is synthesized also remains associated with the plasma membrane and induces changes in local tissue without producing systemic responses.   IL-1 affects mesenchymal tissue remodeling where it contributes to both destructive and repair processes.   IL-1 activates lymphocytes and plays an important role in the initiation

15   of the immune response.   A few IL-1 receptors have been identified, but their affinities often do not match the potency of the biological response.   The most consistent property of IL-1 is up-regulation of cellular metabolism and increased expression of several genes that encode biologically active molecules.

IL-1 is a highly inflammatory molecule that stimulates the production of

20   arachidonic acid metabolites.   IL-1 also acts synergistically with other cytokines, particularly tumor necrosis factor.   The multitude of biological responses to IL-1 is an example of the rapid, adaptive changes that take place to increase the host defen- sive mechanisms.   IL-1 mediates several components of the acute phase response to infection and injury.   The most dramatic biological property is its ability to increase

25   arachidonate metabolites in a variety of cells including PGE in the brain, fibroblasts, synovial cells, and chrondrocytes; in addition, IL-1 reduces lipoxygenase products in lymphocytes and other cells.

IL-1 has been cloned and there are two forms of the molecule.   In the predominant form of IL-1 (IL-1ß) from human monocytes pI = 7.0. Il-β is initially

30   synthesized as a precursor molecule (31 kdal). A minor form (less than one hundredfold) also exists (IL-1α, pI=5). IL-1 seems unique among the lymphokines and monokines in that there is no signal peptide sequence for cleavage.   Depending on the stimulus, intracellular levels of precursor or processed IL-1 can be high. Precursor IL-1 is cleaved into a 17.5 kdal peptide, which is the dominant

35   extracellular form. IL-1 induces prostaglandins and lymphocyte activation as well as many other biological activities.   These include PGE production, protease release

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from synovial cells and chrondrocytes, bone resorption, acute phase protein synthesis, and other effects.

It has been reported that a fragment of IL-1ß contains an active region which is responsible for the immunostimulatory, immuno-restorative and antitumoral

5   properties of IL-1. This region corresponds to residues 163-171 of the IL-1ß molecule and although it is believed to be an active site, it is not associated with any of the inflammatory or pyrogenic properties of IL-1ß. It is believed that an active region other than the 163-171 sequence is responsible for the role IL-1 plays in the inflammation process. The identification of the 163-171 sequence as an active region

10   was first reported by scientists working at the Sclavo Research Center in Siena, Italy. Accordingly, the nine residue sequence is often referred to as the "Sclavo" region. However, as used herein, the terms "Sclavo region", "Sclavo fragment" and "Sclavo peptide" are meant to more broadly define peptides including the 163-171 peptide and those modeled after the nine amino acid sequences of IL-1ß.

15   Interleukin-1 activity is suppressed when IL-1 inhibitors are present. Several IL-1 inhibitors exist and are useful tools in understanding the mechanisms which regulate the action of IL-1. Since the cytokine Interleukin-1 is one of the major mediators of tissue destruction and chronic inflammation, IL-1 inhibition can be used in treating inflammatory diseases. IL-1 inhibitors can also be used as immuno-

20   suppressant molecules since IL-1 plays a central role in the immune response by stimulating proliferation and differentiation of T and B lymphocytes.

The present invention provides an improved Interleukin-1 inhibitor. According to the present invention, a gene for a naturally occurring IL-1 inhibitor has been isolated and sequenced and the DNA sequence encoding the "Sclavo"

25   region has been inserted into the IL-1 inhibitor gene. The resulting modified IL-1 inhibitor has a greater affinity for the IL-1 receptor and the desirable activities of IL-1ß derived from the Sclavo region active site.

INFORMATION DISCLOSURE

PCT International Application Number PCT/US89/02275 discloses the amino

30   acid sequence for a naturally occurring Interleukin-1 inhibitor and the DNA sequence for the gene which encodes the naturally occurring inhibitor. One of the amino acid sequences disclosed is identical to the amino acid sequence encoded by the DNA used as starting material used in the present invention. Methods for production of the inhibitor using recombinant DNA technology are disclosed.

35   PCT International Application Number PCT/US88/02819 published 9 March 1989 discloses an Interleukin-1 Inhibitor purified from urine which is characterized

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by its inhibitory activity as measured by several assays. The IL-1 Inhibitor disclosed has a pI of 4.7.

Antoni, G. et al., J. Immunol. Vol. 137, 10:3201-3204 (1986) disclose a nine residue fragment of human IL-1ß modeled after residues 163 to 171 of naturally

5   occurring human IL-1ß. This nine residue fragment, VQGEESNDK, is reported to show high T cell activity capacity as shown by its ability to stimulate murine thymocyte proliferation and its ability to reduce Interleukin-2 production in spleen cells. However, it is reported that the fragment does not have the capacity to induce prostaglandin production in vitro and does not have in vivo pyrogenic activity.

10   Nencioni, L. et al., J. Immunol. Vol. 139, 3:800-804 (1987) disclose that the nine residue fragment derived from human IL-1ß residues 163-171 enhances antibody responses to both T helper-dependent and T helper-independent antigens. It was shown that the nine residue fragment enhances T helper-dependent antibody response as evaluated in the hemolytic plaque assay of spleen cells from mice

15   immunized with sheep red blood cells. It was also shown that the nine residue fragment was able to enhance the in vivo immune response to a T helper-independent antigen, the poorly immunogenic polysaccharidic antigen from Streptococcus pneumoniae type III, S III. In both cases the enhanced response induced by the nine residue fragment was qualitatively equivalent of that produced

20   by human recombinant IL-1ß.

Boraschi, D. et al., J. Exp. Med 168:675-686 (1988) disclose that the nine residue human Interleukin-1ß fragment derived from residues 163-171 shows immunostimulatory, immunorestorative, antitumoral, and radio-protective capacities comparable to those of the entire protein. It is additionally reported that the nine

25   residue fragment does not produce any of the inflammatory-related and toxic in vivo effects shown by IL-1ß.

Frasca, D. et al, J. Immunol. Vol 141 8:2651-2655 (1988) disclose that the nine residue Interleukin-1ß fragment displays immunorestorative properties similar to those of human recombinant Interleukin-1ß. It is reported that the 163-171

30   peptides restored T cell functions in mice immunocompromised by aging and irradiation. Although the optimal immunorestorative doses of 163-171 peptides were several orders of magnitude higher than those of human recombinant Interleukin-1ß, it is reported that the nine residue fragment displayed a complete lack of IL-1-like inflammatory and toxic effects.

35   Forni, G. et al., J. Immunol. Vol. 141 2:712-718 (1989) disclose that the nine residue 163-171 fragment is less effective than recombinant human IL-1ß in

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triggering lymphokine-activated tumor inhibition (LATI) of a poorly immunogenic fibrosarcoma (CE-2) of BALB/c mice. However, the nine residue peptide does induce a limited LATI and, under certain conditions described in the reference, induced LATI can be increased when injections of the peptide are made with mice challenged

5   with tumor cells admixed with non-reactive lymphocytes.

Boraschi, D. et al., J. Immunol. Vol. 143 1:131-134 (1989) disclose that the synthetic nine residue peptide possesses the immunostimulatory but not the pyrogenic activity of the mature IL-1ß in vivo. A monoclonal antibody (mAb) raised against the nine residue peptide is disclosed. This mAb could effectively recognize

10   human recombinant IL-1ß in RIA and immunoblotting. In vivo, the mAb was able to selectively inhibit the immunostimulatory activity of IL-1ß but could not affect the fever-inducing capacity of IL-1ß.

Peppoloni, S. et al., Nat. Immuno. Cell Growth Regul. 8:10-19 (1989) disclose that the nine residue 163-171 peptide increases the natural killer activity against

15   K562 leukemia cells while the presence of human Interleukin-1ß does not confer a similar increase in natural killer activity. The increase in tumor cell lysis could not be ascribed to the cytolytic activity of the synthetic fragment on target cells, since the peptide caused no direct lysis of various tumor cell lines. Although the peptide enhanced a natural killer cytotoxicity of peripheral blood mononuclear cells, highly

20   purified large granular lymphocytes were not susceptible to its inhibitory effect. Furthermore, the addition to the cultures of antibodies to human Interleukin-2 completely blocked the 163-171 peptide-induced boost of natural killer cytotoxicity.

Amento et al., Proc. Natl. Acad. Sci. USA, 79:5307-5311 (1982), disclose a soluble factor produced by human cell line U937. This soluble factor inhibits IL-1-

25   induced T-cell activation. The molecular weight of this factor is reported to be between 45-60 kdal.

Liao et al., J. Exp. Med. 159:126-136 (1984) teach the identification of an IL-1 inhibitor found in urine from febrile patients. The IL-1 inhibitor disclosed by Liao et al. is between 20-40 kdal. Liao et al. note that the evidence suggests that the

30   molecule is a protein or a glycoprotein but that the evidence is insufficient to support such a statement without additional information.

Scala et al., J. Exp. Med. 159:1637-1652 (1984) disclose a soluble factor derived from the ROHA-9 cell line (a human Epstein-Barr virus-transformed lymphoblastoid cell line) which resembles monocyte-derived human IL-1 inhibitor.

35   Additionally, Scala et al. disclose the presence of a co-existent inhibitory factor. This inhibitory factor has a molecular weight of 95 kdal.

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Arend et al., J. Immunol. 134:6 (1985) teach an inhibitor of IL-1 interaction with chondrocytes or thymocytes which is produced by human monocytes cultured on adherent human complexes or antibodies. The molecular weight of this factor is approximately 22 kdal.

5   Seckinger and Dayer, Ann. Inst. Pasteur Immunol., 138 (3):486-488 (1987) discuss various IL-1 inhibitors. Several inhibitors are disclosed which have been found in urine from highly febrile patients. Among the inhibitors are those having molecular weights of 30-35 kdal, 85 kdal, and 18-25 kdal. The IL inhibitor of molecular weight 18-25 kdal is an immuno-suppressant glycoprotein isolated from

10   urine of pregnant women. Other IL-1 inhibitors disclosed in Seckinger and Dayer are the 22 kdal molecule reported by Arend, et al. which is derived from human monocytes stimulated by adherent immune complexes. A 95 kdal molecule derived from human monocytes stimulated by cytomegalovirus is also disclosed. Additionally, an IL-1 inhibitor with a molecular weight of about 95 kdal derived

15   from human macrophages exposed to influenza and syncytial virus and from human virus-infected B cells is reported.

Hannum, C. H. et al., Nature 343:336-340, January 1990, disclose three Interleukin-1 inhibitors produced by human monocytes. Partial protein sequence data indicates the three isoforms of a single protein.

20   Eisenberg,, S. P. et al., Nature 343:341-346, January 1990, disclose cDNA clone for an Interleukin-1 receptor antagonist produced by human monocytes induced with adherent IgG. The cDNA disclosed is cloned to E. coli where it is expressed to yield Interleukin-1 inhibitor.

SUMMARY OF THE INVENTION

25   The present invention provides an improved IL-1 inhibitor. According to the present invention, an Interleukin-1 Receptor Antagonist Protein (IRAP) which functions as an IL-1 inhibitor is modified by the addition of a "Sclavo peptide". The present invention relates to a DNA molecule which encodes a biologically active Interleukin-1 inhibitor containing a Sclavo region. Furthermore, the present

30   invention relates to such a DNA molecule being incorporated in an expression vector and to a suitable host transformed with an expression vector that contains the DNA molecule encoding IL-1 inhibitor. The present invention relates to a method of producing an improved biologically active IL-1 inhibitor by transforming a suitable host with an expression vector which contains a DNA molecule encoding an

35   improved IL-1 inhibitor and culturing the transformed host cells in conditions promoting expression.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention comprises a polypeptide which contains the amino acid sequence of IRAP augmented with the addition of a "Sclavo peptide". The amino acid sequence for naturally occurring IRAP derived from U937 cells is extremely

5   similar to the amino acid sequence for Interleukin-1ß (IL-1ß). This similarity is demonstrated by the presence of identical residues or conservative substitutions of residues at corresponding regions of the two molecules throughout the two sequences. Accordingly, it is thought that the similarities of the sequences are responsible for IRAP's ability to bind to the IL-1ß receptor but the differences

10   between the two sequences are responsible for the fact that IL-1ß is an agonist while IRAP is an antagonist. Chart 1 shows a comparison between the IL-1ß amino acid sequence and the IRAP amino acid sequence. Asterisks (*) beneath the sequence represent identical residues. Periods (.) beneath the sequence represent residues where a conservative substitution has occurred.

15   One difference between IL-1ß and IRAP is that the IRAP molecule is missing residues corresponding to residues 163-171 of IL-1ß. This region of the IL-1ß molecule is believed to be responsible for the immmunostimulatory, immunorestorative, antitumoral and radio-protective properties of IL-1ß. It is also believed that this region is not responsible for any of the inflammatory-related,

20   pyrogenic or toxic effect characteristics of the naturally occurring agonist. Thus, the 163-171 region is thought to be an active region of IL-1ß but not one responsible for the molecule's role in the inflammation process.

Chart 1 shows a comparison between the IL-1β amino acid sequence and the IRAP amino acid sequence. Chart 2 shows the nucleotide and amino acid sequence

25   of IRAP and Chart 3 shows the amino acid sequence of IRAP.

The present invention uses the following formulae as a basis for constructing molecules:

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     IRAP  Residues 76-85
30      76   77   78   79   80   81   82   83   84   85
         ↓    ↓    ↓    ↓    ↓    ↓    ↓    ↓    ↓    ↓
         I    E    P    H    A    L    F    L    G    I


     Sclavo Peptide Residues 1-9
35       1    2    3    4    5    6    7    8    9
         ↓    ↓    ↓    ↓    ↓    ↓    ↓    ↓    ↓
         V    Q    G    E    E    S    N    D    K
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According to the present invention, an improved IRAP is provided by inserting a peptide sequence modeled after amino acid residues 163-171 of IL-1ß into the corresponding region of the IRAP molecule. The region of the IRAP molecule where the Sclavo peptide is inserted is at the position occupied by amino acid

5   residue 76-79 as shown above and in Chart 3. IRAP residues 76-80 correspond to the 163-171 region of IL-1ß (Scalvo 1-9). Substitutions, deletions and insertions of amino acids may be made throughout the molecule following Dayhoff's rule (Dayhoff, M., Atlas of Protein Sequence and Structure 5:124, 1972). Substitutions may be made within the Sclavo peptide and, in particular, residue 3 may be

10   substituted with any amino acid residue. However, proline may not be substituted for Sclavo peptide amino acid residues 6, 7 or 8. Additionally, tenth and eleventh residues may be added to the Sclavo peptide. Furthermore, any of IRAP amino acid residues 76-85 may be deleted.

In particular, IRAP amino acid residues 76-79 may be deleted and the Sclavo

15   peptide inserted. IRAP amino acid residue 80 may be substituted by a conservative replacement. Sclavo amino acid 3 may be substituted with any amino acid residue. Three improved IRAP molecules are disclosed: MIRAP-1; MIRAP-2; and, MIRAP-3. MIRAP-1 represents the preferred embodiment.

To depict an improved IRAP according to the present invention, amino acid

20   sequences can be written which assign the designation "IRAP residue 76" or "76" and "IRAP residue 85" or "85" although more than 9 residues may lie between the two marked residues. Rather, 76 and 85 serve as reference points to the natural IRAP sequence which is shown in Chart 3. For convenience, improved IRAP molecules may be depicted as short sequences starting with a residue labelled 76

25   and ending with a residue labelled 85. According to this representation, residues 26-75 are present in the molecule adjacent to the residue designated 76. Residues 86-177 of natural IRAP are present in the molecule adjacent to the residue designated 85. The residues depicted between 76-85 in the improved IRAP represent the novel portion of the molecule which is responsible for the improvement over natural IRAP.

30   In Example 9, construction of three embodiments of an improved IRAP is disclosed. These three embodiments may be depicted as follows:

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MIRAP-1                 76                              85
                         ↓                               ↓
35                      V Q W E E S N D K A L F L G I
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MIRAP-2                      76                         85
                             ↓                          ↓
                             V Q G E E S N D K A L F L G I

5   MIRAP-3                  76                         85
                             ↓                          ↓
                             I E P H E S N D K I A L F L G I
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In each embodiment residues 26-75 of IRAP precede the residue labelled 76
10   and residues 86-177 of IRAP follow the residue labelled 85.

This improved IRAP possesses an enhanced ability to bind to the IL-1
receptor. The desirable traits of IL-1ß which are derived from the active site at the
Sclavo region are combined with IRAP's ability to block IL-1 binding to the IL-1
receptor. Thus, an improved IRAP is produced. The improved IRAP has IL-1
15   blocking ability and immunostimulatory activity.

As used herein, the term "Sclavo peptide" and "Sclavo region" are
interchangeable and refer to a peptide modeled after the nine amino acid peptide
found at residues 163-171 of human IL-1ß. "Sclavo peptide" or "Sclavo region"
include those peptides which comprise conservative substitutions of the 163-171
20   peptide of IL-1ß. The 163-171 peptide of IL-1ß consists of the amino acid sequence
VQGEESNDK. Each amino acid residue may be substituted with a conservative
equivalent except proline may not be used in Sclavo positions 7, 8 or 9 and the third
residue, G, may be substituted with any amino acid.

To construct the improved IRAP, the gene for IRAP is isolated and the cloned
25 · into an appropriate vector so that genetic manipulations may be performed. A
series of oligonucleotides are constructed which encode peptides modeled after the
IL-1ß 163-171 peptide. These oligonucleotides were inserted into the portion of the
IRAP gene which encodes residues 76-80 of IRAP.

The gene encoding IRAP was isolated by screening a cDNA library made
30   from the RNA of U937 cells. The cDNA library was screened with oligonucleotide
probes which hybridize to nucleotide sequences that can encode an amino acid
sequence known to exist within the IRAP protein. The known amino acid sequence
upon which the probes were modeled was discovered by sequencing a portion of the
IRAP protein isolated from the cultured cells. The nucleotide sequence which
35   encodes IRAP including the sequence that encodes the 25 amino acid signal
sequence is shown in Chart 2 and the amino acid sequence of the IRAP protein
including the 25 amino acid signal sequence is shown in Charts 2 and 3. When

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expressed in nature, the protein is 177 amino acids. Residues 1-25, the signal
sequence, are cleaved off and the mature protein is produced.

Once the gene is isolated, it may be used to produce IRAP using techniques
well known to those having ordinary skill in the art. The gene may be inserted into
5   an expression vector and the expression vector may be used to transform a suitable
host. The transformed host may be cultured in conditions which promote expression
of the IRAP gene and, thus, production of the IRAP protein.

Improved IL-1 inhibitors contain peptides modeled after the 163-171 peptide.
The IL-1ß-derived Sclavo sequences is VQGEESNDK. Each of the residues may be
10 replaced with a conservative substitution except proline may not be used in Sclavo
positions 7, 8 or 9. Additionally, the amino acid at position 3, G, may be replaced by
any amino acid. Furthermore, additional amino acid residues may be added except
proline. In order to produce these peptide sequences within a protein molecule, the
nucleotide sequence encoding the peptide sequence is synthesized as an
15 oligonucleotide. This oligonucleotide is inserted into the gene encoding IRAP. The
recombinant gene is then placed in an expression vector and the improved IRAP will
be produced.

Example 1   IRAP Production from U937 Cells Treated with GM-CSF

The established cell line U937 (ATCC) cells was grown in RPMI 1640
20 medium containing 10% fetal calf serum (FCS; Sterile Systems, Logan, UT), 100
µg/ml streptomycin (GIBCO, Grand Island, NY), and 100 units/ml penicillin
(GIBCO). The cell line U937 was originally described by Sundstrom and Nilsson,
Int. J. Cancer, 17:565-577 (1976). Cells were harvested and washed in phosphate
buffered saline (PBS) and reseeded in the same medium containing 100 nM PMA at
25 2×10^5 cells/cm^2. The cells were kept in this medium at 37°C for forty-eight hours,
at which point most of the cells adhere to the plate surface. The medium was
removed, the cells were washed with PBS and refed with RPMI 1640 containing 1%
FCS and 100 units/ml GM-CSF (Amgen, Thousand Oaks, CA). The cells were
incubated in this medium for 24-120 hr and then harvested. Cell debris was
30 removed by high speed centrifugation. Supernatants thus obtained were assayed for
inhibiting activity.

Example 2   IRAP Production from U937 Cells Treated with IL-4

After PMA differentiation of U937 Cells, 100 units/ml of recombinant human
Interleukin-4 (IL-4, Genzyme, Boston, MA) was added to RPMI 1640 medium
35 containing 1% Hyclone serum, Penn/Strep and glutamine (Std. Protocol reagents).
Measurement of IRAP messenger RNA and assay for the IRAP protein (either the

1A5 or receptor binding assay) indicated that the kinetics of induction were roughly twice as fast as that seen with GM-CSF. That is, the 1/2 maximal levels of both protein and RNA were reached in less than 10 hours as compared to 24 hours being the norm for GM-CSF. At 24 hours, protein secreted in the supernatant after IL-4

5 treatment was nearly double that measured in GM-CSF supernatants at 48 hours.

Thus, the IL-4 induction of IRAP inhibitor in U937 was more rapid and higher inhibitor levels were reached than by any previous method of induction.

Example 3   Characterization   by Inhibitory Activity Assay by the
1A5/HT2 T-Cell IL-1 Antagonist Assay

10 The assay used to determine the presence of an IL-1 inhibitor has been described in Tracey et al., Immunopharmacology, 15:47-62 (1988). This assay, the 1A5/HT2-T-Cell IL-1 Antagonist Assay, confirms the presence of a product capable of inhibiting IL-1 activity. In order to determine IL-1 inhibitor activity, supernatants recovered from harvesting the treated cells were added with IL-1 and phytohema-

15 glutin (PHA) to a murine T-cell line which produces IL-2 when exposed to IL-1 and PHA. Thus, if the supernatants added contain an IRAP, the inhibitor will block production of IL-2. The production of IL-2 can be determined by adding the medium recovered from the treated murine T-cells to an IL-2 dependent murine T-cell line. If IL-2 has been produced by the stimulated cells, it will be present and available for

20 the IL-2 dependent cells to use. If the production of IL-2 has been blocked by the presence of the IL-1 inhibitor, no IL-2 will be present and the IL-2 dependent cells will die. To test the viability of the IL-2 dependent cells, radiolabelled thymidine is added to medium. If the IL-2 dependent cells are alive and growing, the radio-nucleotide will be incorporated into the cells. If the cells are dead, all the radiolabel

25 will be washed away when the medium is removed. Thus, when the cells are harvested and placed in a scintillation counter, the presence of the radiolabel can be detected. Accordingly, the presence of IL-2 can be established and, therefore, the presence of IRAP can be determined.

Murine T cell line LBRM-33-1A5 (ATCC) cells were maintained at 37°C in

30 5% CO_2 atmosphere in RPMI 1640 with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. IL-2 dependent murine T cells HT-2 as described in Watson, J., J. Exp. Med., 150:1510-1519, (1979) were maintained in continuous culture in culture medium further supplemented with 10 mM HEPES buffer (GIBCO), 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak, Rochester, NY) and 15% supernatant from

35 concavalin A-stimulated rat spleen cells. This supernatant, used as a source of IL-2, was generated by stimulation of Wistar-Sprague rat spleen cells (Charles River,

Cambridge, MA) at 5×10^6 cells/ml in culture medium with 5 μg/ml concavalin A (Pharmacia, Piscataway, NJ).

On Day 1 of a three day procedure, 100 μl (1×10^6 cells/ml) of a subcloned line of murine T cell line 1A5 with 4 μg/ml PHA was placed in a V-bottom well on a

5 96 well plate with 50 μl supernatant and 50 μl of IL-1 (0.01-0.02 units/ml) for 24 hours after which the contents were centrifuged for 6 minutes at 300 x g and 4°C.

On Day 2, in a 96 well flat-bottom plate, 100 μl of Day 1 supernatant was added to 100 μl HT-2 cells (1×10^5 cells/ml) and maintained for 24 hours.

On Day 3, 50 μl of ^{3}H-Thymidine (2 Ci/mmol; Amersham, Arlington Heights,

10 IL) diluted to 10 μCi/ml in medium was added to each well. After 4 hours, the cells were harvested and placed in a scintillation counter. Absence of radioactive isotopes in a sample indicates that the cells from which the sample was derived are not alive and therefore IL-2 was not produced and IL-1 inhibitory activity is indicated. As a control, on day 1, 50 μl of medium was added to the well instead of supernatant from

15 harvested cells. Thus, no inhibitor was added, IL-2 should be produced and HT-2 cells should live and take up the radioisotope. The scintillation counter results for the control indicate no inhibitory activity. The amount of inhibitory activity in a sample can be determined relative to this control.

Example 4   Characterization by Inhibitory Activity Assay by the Receptor

20   Binding Assay

The presence of IRAP was confirmed by the Receptor Binding Assay to be a receptor level inhibitor. This assay demonstrates that IRAP competes with radioactively labelled IL-1 to bind to cells known to have receptors to IL-1.

The whole cell IL-1 competition assay using YT-NCl cells and [^{125}I]-labelled

25 IL-1ß was used to follow purification of the U937 IL-1 inhibitor. Receptor binding assays were conducted in 96-well microtiter plates and contained 1 million YT-NCl cells, 50 pM [^{125}I]-IL-1ß, and an aliquot of "inhibitor" sample in a final volume of 200 μl. The cells were incubated at ambient temperature for one hour and unbound radioactivity was removed by filtration. Non-specific binding was determined by

30 including 50 nM unlabelled IL-1 in the assay, and was generally less than 10%. The presence of IRAP was detected by a decrease in the amount of labelled [^{125}I]-IL-1 bound to the cells.

Example 5   Purification and Characterization of IRAP from U937

                    Cells

35   The 1A5 and Receptor binding assay are both used throughout the characterization and purification procedures to confirm the presence of IRAP in the

various samples. IRAP is then purified from samples found to contain inhibitor activity and characterized using various protein chemistry techniques. The sample in medium is concentrated by ultrafiltration (Amicon, YM5 membrane, molecular mass cutoff 5000 daltons).

5   Concentration by Ultrafiltration

IRAP, produced in the culture media of U-937 cells stimulated with PMA and GM-CSF, was present in a large volume of solution with a relatively high concentration of albumin as the principle contaminant. In order for the purification to be successful, it was necessary to remove gram quantities of albumin and other

10 serum proteins from microgram quantities of the desired protein. As a first step, the supernatants were concentrated by ultrafiltration. This step removed PMA and peptides having masses less than 10,000 daltons, which might influence the inhibitor bioassays.

Protein solutions were concentrated in ultrafiltration cells having 2 liter and

15 400 ml volume capacity (Amicon Corp., Danvers, Mass.) at 4°C by nitrogen pressure. Added to stabilize the inhibitor protein during processing and to reduce proteolysis which was a likely causative factor of reduced yields was 0.2 mM phenylmethanesulfonylfluoride (PMSF).

A portion of the concentrated material was analyzed to determine certain

20 physical characteristics of IRAP. Analytic techniques performed included analytic gel filtration chromatography, isoelectric focusing, native polyacrylamide gel electrophoresis profiles and mono Q fractionation.

The remainder of the material was purified using protein preparation techniques. These included preparative gel filtration using a Superose 12 FPLC

25 prep column, two procedures using TSK Bio-Sil 125 HPLC columns, and a final purification step using a C4 reverse phase HPLC column. Between preparative procedures, the presence of IRAP was confirmed using 1A5 and Receptor Binding assays. SDS PAGE profiles were performed on essentially pure IRAP to determine physical properties.

30 Characterization

Gel Filtration Chromatography Profiles

A small aliquot (0.5 ml) of the above concentrate was injected onto an analytical Superose 12 FPLC column equilibrated with 0.1 M Tris HCl, pH 7.5. Fractions of 1.0 ml were collected isocratically at a flow rate of 0.5 ml/minute at

35 room temperature. Fractions (1.0 ml) were assayed for protein using the Bradford dye-binding method and were then assayed for inhibitory activity. Marker proteins

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having known molecular masses were run separately in an identical manner. These proteins included BSA (67 kdal), carbonic anhydrase (30 kdal), myoglobin (17 kdal), and aprotinin (6,600 daltons). A standard curve was constructed by linear regression analysis of the line generated of log molecular mass versus retention

5 time.

The molecular mass of the inhibitor activity on Superose 12 FPLC columns was 25,000 daltons, with a range of error of 19,000 through 32,000 daltons.

Native Polyacrylamide Gel Electrophoresis Profiles

The 6% polyacrylamide gels (37.5 acrylamide:bis-acrylamide ratio) were

10 polymerized and run as described by Laemmli (Nature, 227:680-685 1970), except that sodium dodecyl sulfate was omitted. Samples containing IRAP were prepared for electrophoresis by addition of glycerol and bromophenol blue (Aldrich, Milwaukee, WI) which was used as a dye front indicator. Gels are run for 12-16 hours at 4°C at low current and voltage.

15 Two peaks of activity were observed. The standard used to measure the distance travelled by the protein was the ratio of distance travelled by the peak to the distance travelled by the dye front. The first peak travelled 53% of the distance travelled by the dye. The second peak travelled 61% of the distance travelled by the dye. Both peaks represent the inhibitor, either as monomer and dimer forms under

20 native conditions, or as two populations of proteins having common molecular masses but differing in glycosylation.

Anion Exchange Chromatography Profiles

IRAP was shown to bind to anion exchange matrices at pH values greater than 7.5. However, optimal results were obtained using quaternary anion exchange

25 columns, such as Mono Q or QAE Fast Flow matrices (Pharmacia) rather than by classical DEAE Cellulose supports. Elution from the former columns was achieved by utilization of a linear salt gradient with fractions collected and assayed. In the column gradient elution procedure, the starting buffer was 25 mM Tris HCl, pH 8.5, and 10% glycerol, while the termination buffer was the same buffer including 1 M

30 NaCl. Samples were prepared for column chromatography by dialysis against the starting buffer for at least a three hour period at 4°C in dialysis bags having a 5,000 dalton exclusion limit. The typical column was 1.6 x 50 cm (dimensions) for QAE Fast Flow and 0.5 x 5 cm for the Mono Q column (Pharmacia-LKB).

Inhibitory activity was associated with a protein fraction which elutes in the

35 gradient at a position corresponding to a 125-160 mM NaCl concentration.

Isoelectric Focusing

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Native isoelectric focusing was conducted in 6% polyacrylamide gels [37.5:1.0 acrylamide:bis- acrylamide ratio] in a narrow range pH gradients established with 2.0% (w/v) Bio-Lytes, pH 4-6, supplemented with 0.5% (w/v) Bio-Lytes, pH 3-10. Gels were polymerized in 0.5 cm diameter borosilicate glass tubes (Bio-Rad) using

5   ammonium persulfate, and allowed to stand at room temperature for at least 1 day prior to use. Samples of IRAP were mixed with 1 volume of 50% glycerol containing 0.05% methyl red, applied to the high pH end of the gradient (top), and electrofocused at 100 volts (constant) for 24 hours at 4°C. The anodic and cathodic solutions were 0.1 M phosphoric acid and 0.1 M ethanolamine, respectively.

10   Following electrophoresis, gels were removed by water rimming, and cut into 2 mm sections. The pieces were placed in 1.5 ml microfuge tubes containing 0.25 ml water. After 4 hours incubation, pH readings were taken with a Beckman micro-electrode. These extracts were also analyzed by the cellular bio-assay and receptor binding assay as described above.

15   Using isoelectric focusing gel electrophoresis in polyacrylamide, four peaks of inhibitory activity were observed by bioassay and receptor binding assay. Of these four pI forms, the pI 5.18 species has the highest activity. These data were also verified by flat-bed isoelectric focusing in Ultradex. The purified IL-1 inhibitor was analyzed by 2-D polyacrylamide gel electrophoresis. A principle spot was observed

20   following Coomassie Brilliant Blue G-250 staining, having the coordinates of pI = 5.1 and mass = 25,000 daltons. A second spot, of lower intensity, is noted at the same mass position (y-axis), but differing in the observed pI. To verify that these spots are truly IRAP, the fractions from analytical isoelectric focusing gels were analyzed by 2-D PAGE. The results of this analysis showed that the primary

25   activity corresponded to a spot at 25 kdal and pI = 5.1, while the secondary spot aligned perfectly with a 25 kdal protein having a pI = 4.9. Therefore, it is shown that (a) the inhibitor protein(s) could be seen with Coomassie Brilliant Blue G-250 on 2-D gels even in the crude supernatants, and (b) the 1D IEF profiles of crude supernatant inhibitor, when individual fractions were subsequently analyzed by 2-D

30   PAGE, confirmed the assignments we have made using the purified protein.
Preparative Purification

Preliminary chromatography of inhibitor-enriched samples on TSK Bio-Sil 125 gel filtration columns under low ionic strength did not result in any resolution from the serum protein (especially, albumin) contaminants. Thus, under these

35   conditions, much of the inhibitor activity could be localized in the large albumin peak at 67 kdal. However, the inclusion of 1 M NaCl into the column buffer

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effectively prevented any interaction between inhibitor and the large molecular weight protein contaminants. Using the TSK Bio-Sil 125 column under the high salt conditions, the IL-1 inhibitor was estimated to have a molecular mass of about 25,000 daltons.

5   Since the volume of the preparation was still incompatible with direct runs on the HPLC column after Amicon ultrafiltration, the sample was instead chromatographed on a Superose 12 FPLC column (1.6 X 50 cm) equilibrated with the high salt conditions. (Using the FPLC column system allowed for 2 ml injections per run as compared to 50 μl injections for the HPLC system).

10 Superose 12 FPLC-1

          Two ml aliquots were injected onto a Superose 12 prep-grade FPLC column (1.6 x 50 cm) equilibrated with 0.1 M potassium phosphate, 1 M NaCl, pH 6.0. Fractions of 1 ml were collected at a flow rate of 1 ml/min. Fractions from each series of runs were pooled, and analyzed by the 1A5/HT2 assay (bioassay) and the

15 receptor binding assay described above. Protein assays of the fractions were determined by a protein assay kit (A 595; Bio-Rad Laboratories). Appropriate fractions were collected individually concentrated by Centricon-10 units (Amicon). After analysis by SDS PAGE and bioassay, appropriate fraction concentrates were pooled and further concentrated to 1 ml by Speed Vac centrifugation (Savant).

20 TSK Bio-Sil 125 HPLC-I and TSK Bio-Sil 125 HPLC-II

          Fifty μl at a time of the Superose 12 FPLC concentrated preparation were injected onto a TSK Bio-Sil 125 HPLC column (7.5 x 600 mm), using the same buffer system as described above. 200 μl fractions were collected. Active fractions were determined by receptor binding assay or by 2-D PAGE. Appropriate fractions were

25 pooled and concentrated by Centricon-10 filter units and finally by Speed Vac centrifugation as described previously to a final volume of 0.14 ml.

          Twenty μl at a time of the Bio-Sil 125 HPLC concentrated preparation were injected onto the same column as described above. Active fractions were pooled to give a final volume of 8.5 ml.

30   In summary, the Superose 12 pool concentrate was injected in multiple cycles onto a TSK Bio-Sil 125. This fractionation, because of the precision used in peak selection, resulted in the removal of almost all of the albumin and over a 500-fold purification for this step alone. This step was repeated a second time to remove residual albumin and low molecular weight contaminants. While resulting in a 3-

35 fold enhancement of specific activity, it is conceivable that this second run of the column could have been avoided, since the C4 reverse phase fractionation (next step)

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works effectively on all preparations of the inhibitor having specific activities of at least 100 U/mg.

Finally, the TSK Bio-Sil 125 HPLC pool concentrate was injected onto a C4 reverse phase HPLC column.

5   C4 Reverse Phase HPLC Chromatography Profiles

C4 reverse phase HPLC columns were used to further resolve the inhibitor protein. The resolution capabilities of this column were dependent primarily on the hydrophobic interactions of the protein with the C4 matrix, and the relative affinity of the protein for either the stationary phase (C4) or the mobile phase (0.1% TFA,

10   pH 2, variable concentrations [v/v] of acetonitrile). The columns used were 1 x 25 cm dimensions with starting solutions of 0.1% TFA and 0% acetonitrile. The gradient of acetonitrile is incremented from 28-32% over a 52 minute period. Other variations can also be used in these test runs. Under all conditions, the same elution position was achieved. The protein was injected onto the column in the

15   buffer it was stored in prior to the run with no requirement for a pre-incubation or pre-equilibration step. Fractions were collected, taken to dryness with a SpeedVac (Savant) centrifugal evaporator, and redissolved in a minimal volume of tissue culture media for biological activity measurements.

IRAP elutes at a position corresponding to an acetonitrile concentration of

20   between 30-31% (v/v). The bioactivity was recovered following this operation. The elution position for this protein is a physical characteristic which is highly reliable (5% maximal error in elution position).

SDS Polyacrylaminde Gel Electrophoresis Profiles

One dimensional SDS PAGE was performed using 14% - 20%, preferably

25   18%, gels in a Protean II system or in a mini Protean II system (Bio-Rad), according to the method of Laemmli. Before electrophoresis, samples were diluted 1:1 with denaturation buffer (2% SDS, 12.5% glycerol, 0.125 M Tris HCl, pH 6.8, and 3% ß-mercaptoethanol), and heated in a boiling water bath for 2 minutes. Electrophoresis was conducted at constant power (5 watts/gel) for 1 hour (mini gels) or 6 hours

30   (regular gels) at room temperature and terminated when the dye front (bromophenol blue) is 0.5 cm from the bottom. The completed gels were fixed in 50% ethanol and 10% acetic acid, and stained with Coomassie Brilliant Blue G-250 or with a commercially available silver staining kit (Bio-Rad). Photos of gels were made using Type 665 film (Polaroid).

35   Two dimensional SDS PAGE was also done. Samples to be analyzed were desalted by microdialysis (Enprotech) against deionized water. Two dimensional

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electrophoresis was conducted using 1 mm diameter capillary-tube gels (7% polyacrylamide) in the first dimension (4-8°C; 1200 V, constant voltage) for 10-12 hours. Ampholines having a pH range of 4-6 were used (Bio-Lytes, 2% w/v, Bio-Rad Laboratories). Focused sample gels were electrophoresed onto a second dimension of

5   18% polyacrylamide (9 cm x 7 cm) (0.1% SDS) according to the method of Laemmli.

Initial Characteristics (2D Gel Coordinates) of IRAP show that crude U937 IL-1 inhibitory activities migrated at 4 different pI's: 4.6, 4.9, 5.1, and 5.5 (narrow range pH 4-6 ampholyte gel system). The heterogeneity was due to glycosylation, rather than differences in amino acid sequence. The major form of the U937 derived

10   protein has a native mass of ~25 kdal as determined by size exclusion chromatography. An analytical sample of IRAP was purified to homogeneity, and run on 2D PAGE. Two spots (25 kdal; pI's of 4.9 and 5.1) were observed using Coomassie Brilliant Blue G-250 staining.

Deglycosylation of the Protein with N-Glycanase

15   The protein (both pI forms, 4.9 and 5.1) was subjected to incubation with N-glycanase to determine if it is glycosylated. To 10 μl of inhibitor protein were added 10 μl of 1% (w/v) SDS. This solution was heated for 2 minutes in boiling water. To the resulting solution was added NaPO4 (0.14 M final), pH 8.0, and NP-40 (1.0%). As a result the NP-40 to SDS ratio was at least 7:1. N-glycanase (excess) was added

20   and incubation allowed to proceed for overnight at 37°C. Controls were run using ovalbumin, RNAse B and a2-macroglobulin to determine the amount of N-Glycanase needed for complete conversion of a specific amount of total glycoprotein.

Following the reaction with N-glycanase, a protein band migrates with a mass of 22 kdal, indicating that at least 3 kdal of the mass of the apoprotein are

25   carbohydrate.

A characteristic of IRAP includes a capability of retaining bioactivity following treatment with sodium dodecyl sulfate detergent, guanidine hydrochloride denaturant, trifluoroacetic acid, and acetonitrile; indicating an inherent stability to harsh chemicals.

30   Example 6   Isolation of the Gene Encoding IL-1 Inhibitor Protein

To obtain sequence information for use in oligonucleotide probe construction and peptide synthesis, we investigated several avenues for sample preparation: (a) N-terminal sequencing of the intact 25 kdal protein; (b) Endo Lys C Digestion of the Native protein followed by C18 RPC HPLC and peptide sequencing of selected

35   peaks; and (c) Endo Lys C Digestion of S-Pyridylethylated Inhibitor Protein followed by C18 RPC HPLC and peptide sequencing of selected peaks.

Sequence Methods

         N-Terminal Sequence-Automated Edman degradation chemistry was performed in an Applied Biosystems, Inc., Model 470A gas phase protein sequenator fitted with an on-line Model 120A PTH amino acid analyzer. Data were collected

5   and yields calculated on a Nelson Analytical 3000 Series chromatography system. Results suggested that the N-terminus was blocked (a sequence APHG was observed, but at low yield; identification of protein retained on the disk was performed by amino acid composition). However, this conclusion is speculative as the N-terminus may have been artificially blocked as often observed in low protein isolation

10   conditions.

Peptide Mapping

S-Pyridylethylation procedure

         In the mapping procedure, two avenues were used including one in which a sample first undergoes S-Pyridylethylation. A protein sample (35 μg, ~1.4 nmoles)

15   was first denatured in 6 M guanidine HCl, 250 mM Tris HCl, and 50 mM EDTA, pH 8.5. 1 μl of 2-merceptoethanol (10%, v/v) was added and the solution further incubated at 37°C for 30-40 minutes. One μl of 4-vinyl-pyridine (Aldrich Chemical Co.) was then added and the solution incubated at room temperature for an additional 3 hours. Low molecular weight reagents were removed by dialysis with a

20   Prodialyser (Enprotech) against 2 liters of deionized water over a 4 hour period. The final volume of the reaction product was reduced to 50 μl by Speed Vac centrifugation.

Endoproteinase Lys C Digestion

         Whether using native or S-Pyridylethylated sample, the sample was then

25   subjected to Endoproteinase Lys C Digestion. Three hundred μl of 10 mM Tris HCl, pH 8.7 were introduced to the above solution of S-pyridylethylated inhibitor protein, followed by the addition of 1 unit of reconstituted endoproteinase Lys C (EKC; Boehringer). This solution was incubated at 37°C for 12-14 hours.

         Next, the Endo Lys C generated peptide mixture was resolved on a C18

30   reverse phase HPLC column (4.5 X 150 mm) using a linear gradient from 0 to 36% acetonitrile in 0.1% TFA over a period of 52 minutes. The column was monitored at 206 nm and peptides were collected manually during peak elution. Solvent was removed by vacuum centrifugation (Savant).

Example 7   Identification and Cloning of IRAP Gene

35   One of the peptides sequenced has the amino acid sequence KIDVVPIE where the letters correspond to the one-letter symbol code for amino acids. Degenerate

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oligonucleotides which contained all possibilities represented by the amino acid sequence KIDVVP were synthesized using an Applied Biosystems Synthesizer. These degenerate oligonucleotide pools were decoded by using Northern blot analysis to determine the homology of the degenerate oligonucleotide with the U937

5 mRNA. The degenerate oligonucleotide pool giving the best hybridization signal to the U937 mRNA was shown to be DC-166:

```
        C    C    C      G    C
5'-GG   AC   A A  TC   AT   TT-3'
        T    T    G      T    T
```

10
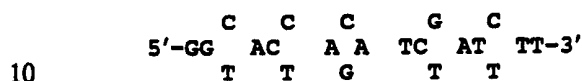
When DC-166 was labelled with τATP^{32} and used to screen a U937 cDNA library which was derived from cells stimulated three days with 50 μg/ml phorbol myristic acetate (PMA), a clone (P5) was isolated and sequenced by the dideoxy NTP

15 method of sequencing. The DNA sequence of the derived insert is set forth in Chart 1.

The human DNA sequence contains about 1782 nucleotides including 5' and 3' nontranslated sequences. The longest open reading frame begins with the fifty third nucleotide and extends through the 584th nucleotide. The protein derived by

20 translation of the nucleotide sequence beginning with the first Met from Nucleotide 53 is set forth in Chart 1. This sequence is comprised of 177 amino acids (including 25 amino acids of a signal peptide). Thus, the mature IRAP is 152 amino acid residue shown in Chart 2 as 26-177. The coding sequence of the IRAP gene for the mature protein is nucleotides 128-584. The human sequence includes one N-

25 glycosylation site at position 377-379 of the DNA sequence (Asn 109).

Example 8   Preparing IL-1 Inhibitor Using Recombinant DNA Technology

E. coli Expression of IL-1 Inhibitor

Starting material was the cDNA clone P5 which has the cDNA inserted into the EcoRI site of the commercially available vector pGEM-2.

30 The ~1.6 kb EcoRI/Klenow/SpeI fragment was isolated from pGEM-2/P5. This fragment contains part of the coding sequence for IRAP, from nucleotide 215 to 584. This fragment was inserted into one expression vector pTrp2-m4 (MKOlsen, SKRockenbach, KACurry and C-SCTomich, J. of Biotechnology 9:179-190, 1989) which was treated with HindIII/Klenow/ClaI, together with two oligonucleotides.

35 The oligonucleotides contain the coding sequence for IRAP from nucleotide 128-214, the translation initiation codon ATG and the ends of ClaI and SpeI. In the oligonucleotide sequence, codon usage is maximized and secondary structure is

-20-

minimized in order to optimize translational efficiency. The resulting vector is named pTrp2-ILinh which carries the IRAP sequence under the control of the E. coli trp promoter and an AT-rich ribosome binding site, in a pBR322 background (Chart 4). DNA sequencing was carried out to confirm the sequence of the region

5   manipulated.

       E. coli K12S carrying pTrp2-ILinh was induced for expression under tryptophan starvation conditions. Cell extracts exhibit inhibitor activity and SDS-PAGE analysis shows a faint band corresponding to the expected size, demonstrating low level of expression.

10   To increase expression, the 1.2 kb EcoRI-BamHI fragment containing the trp promoter, ribosome binding site and the coding sequence for IL-1 inhibitor was isolated from pTrp2-ILinh and cloned into the EcoRI and BamHI sites of pUC19. The resulting vector is called pUC-ILinh (Chart 5).

       E. coli was transformed with pUC-ILinh. Extracts from E. coli K12S (pUC-

15   ILinh) induced for expression by tryptophan starvation shows inhibitor activity. SDS-PAGE analysis of the extracts shows a prominent band at the expected size, indicating high level expression.

Purification of Recombinant IL-1 Inhibitor IRAP

       E. coli K12S (pUC-ILinh) were propagated, induced for expression by

20   tryptophan starvation and centrifuged. The E. coli pellets were resuspended in 10 mM Tris HCl, pH 8.0. The suspension was freeze thawed; first the pellets were frozen immediately in dry ice for 10-15 minutes after which the mixtures were thawed in warm water. The pellets were freeze thawed at least once, preferably more than four times. The supernatant was then collected by centrifugation (Sorvall

25   SS-34, 8°C at 20,000 RPM). This clarified solution was loaded onto a FPLC system equipped with an equilibrated 1 X 10 cm (QAE fast flow; Pharmacia) column in 10 mM Tris HCl, pH 8.0, at a flow rate of 1 ml/min. The retained proteins were resolved with a linear NaCl gradient from 0-300 mM over 82 minutes at 3 ml/min. Fractions containing the 22 kdal apparent M.W. protein (SDS PAGE) were pooled

30   and concentrated and buffer exchanged to 10 mM Tris Hcl, pH 9.0, by Centricon-10 (Amicon). This enriched material was further refined by HPLC on a TSK DEAE 5PW (0.75 X 75 mm) column using a linear NaCl gradient (10-125 mM) in 20 mM Tris HCl, pH 9.0, over a 25 minute period. The pooled material was concentrated by Centricon 10. Other minor contaminants were finally removed by gel filtration on a

35   TSK Bio-Sil 125 HPLC column (0.75 X 600 mm) in 50 mM phosphate, 1 M NaCl, pH 6.8. The purified recombinant protein was stored frozen at -20°C in 20 mM Tris

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HCl, pH 7.2. SDS PAGE with urea indicated the molecular weight of the purified recombinant IRAP protein to be about 17,000 daltons.

Example 9   Construction of Oligonucleotides Encoding Sclavo
                   Peptides

5   In order to construct improved IRAP molecules according to the present invention, manipulations are performed on the IRAP gene to insert oligo nucleotides that encode a Sclavo peptide into the appropriate region of the IRAP gene. The resulting recombinant gene encodes the improved IRAP which is produced when the gene is expressed.

10   To construct oligonucleotides, an Applied Biosystems 380 oligonucleotide synthesizer was used. The following oligonucleotides and their complementary strands were constructed:

#1)   Oligo-1   5'-CCG TCC AGT GGG AGG AGT CCA ACG ACA AAG CTC
                  TGT TCT TGG GAA TC-3'

15   #2)   Oligo-2   5'-CCG TCC AGG GTG AGG AGT CCA ACG ACA AAG CTC
                  TGT TCT TGG GAA TC-3'

#3)   Oligo-3   5'-CCA TTG AGC CGC ATG AAT CTA ACG ATA AAA TCG
                  CTC TGT TCT TGG GAA TC-3'

Oligo-1 encodes the amino acid sequence VQWEESNDKALFLGI.   Oligo-2
20   encodes the amino acid sequence VQGEESNDKALFLGI.   Oligo-3 encodes the amino acid sequence IEPHESNDKIALFLGI.

Example 10   Insertion of Oligonucleotides That Encode Scalvo Peptides into
                    the IRAP Gene

The IRAP gene was isolated from pUC-ILinh by digestion with ClaI and
25   BamH1 and inserted into pGEM2 digested with ClaI and BamH1 forming pGEM2IRAP. To insert the complimented oligonucleotides encoding Sclavo peptides, pGEM2IRAP was digested with KpnI and NcoI, effectively deleting the coding sequence from amino acid residue 75 to 85. The oligonucleotides were inserted into the digested pGEM2IRAP by ordinary ligation, forming pGEM2MIRAP-1,
30   pGEM2MIRAP-2, and pGEM2MIRAP-3, respectively.

Plasmid pGEM2MIRAP-1 contains oligo-1 inserted into the KpnI/NcoI digested IRAP gene construct. The MIRAP-1 gene encodes the natural IRAP amino acid sequence 26-75 followed by VQWEESNDKALFLGI followed by the natural IRAP amino acid sequence 86-177.

35   Plasmid pGEM2MIRAP-2 contains oligo-2 inserted into the KpnI/NcoI digested IRAP gene construct. The MIRAP-2 gene encodes the natural IRAP amino

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acid sequence 26-75 followed by VQGEESNDKALFLGI followed by the natural IRAP amino acid sequence 86-177.

Plasmid pGEM2MIRAP-3 contains oligo-3 inserted into the KpnI/NcoI digested IRAP gene construct. The MIRAP-3 gene encodes the natural IRAP amino

5   acid sequence 26-75 followed by IEPHESNDKIALFLGI followed by the natural IRAP amino acid sequence 86-177.

Example 11   Insertion of Modified IRAP Gene into Expression Vectors

Starting material was the recombinant construct described above: pGEM2MIRAP-1; pGEM2MIRAP-2; and pGEM2MIRAP-3.

10   The ~1.6 kb EcoRI/Klenow/SpeI fragment was isolated from each plasmid respectively. The fragment contains the coding sequence for the improved IRAP, from residue 30 to the end. This fragment was inserted into one expression vector pTrp2-m4 (MKOlsen, SKRockenbach, KACurry and C-SCTomich, J. of Biotechnology 9:179-190,1989) which was treated with HindIII/Klenow/ClaI, together with two

15   oligonucleotides. The oligonucleotides contain the coding sequence for IL-1 inhibitor from residue 26-29, the translation initiation codon ATG and the ends of ClaI and SpeI. In the oligonucleotide sequence, codon usage is maximized and secondary structure is minimized in order to optimize translational efficiency. The resulting plasmid is named pTrp2-MIRAP-1, pTrp2-MIRAP-2, or pTrp2-MIRAP-3, respectively,

20   (referred to herein generally as pTrp2-MIRAP) carries the improved IRAP sequence under the control of the E. coli trp promoter and an AT-rich ribosome binding site, in pBR322 (Chart 6). DNA sequencing was carried out to confirm the sequence of the region manipulated.

E. coli K12S carrying pTrp2-MIRAP was induced for expression under

25   tryptophan starvation conditions. Cell extracts exhibit inhibitor activity and SDS-PAGE analysis shows a faint band corresponding to the expected size, demonstrating low level of expression.

To increase expression, the 1.2 kb EcoRI-BamHI fragment containing the trp promoter, ribosome binding site and the coding sequence for IL-1 inhibitor was

30   isolated from pTrp2-MIRAP and cloned into the EcoRI and BamHI sites of pUC19. The resulting vector is called pUC-MIRAP (Chart 7) (pUC-MIRAP refers to pUC-MIRAP-1, pUC-MIRAP-2 or pUC-MIRAP-3) .

E. coli was transformed with pUC-MIRAP. Extracts from E. coli K12S (pUC-MIRAP) induced for expression by tryptophan starvation shows inhibitor activity.

35   SDS-PAGE analysis of the extracts shows a prominent band at the expected size, indicating high level expression.

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This construction strategy was followed for each of the three MIRAP gene embodiments described above (MIRAP-1, MIRAP-2 and MIRAP-3).

The modified IRAP genes produced improved IRAP. The bioactivity of the product may be determined by in vitro transcription and translation of the gene
5   followed by testing of the product using the 1A5/HT2 Bioassay.

Example 12   Purification of MIRAP Protein

E. coli K12S containing one of pUC-MIRAP-1, pUC-MIRAP-2, or pUC-MIRAP-3 are propagated, induced for expression by tryptophan starvation and centrifuged. The E. coli pellets are resuspended in 10 mM Tris HCl, pH 8.0. The suspension is
10 freeze thawed; first the pellets are frozen immediately in dry ice for 10-15 minutes after which the mixtures are thawed in warm water. The pellets are freeze thawed at least once, preferably more than four times. The supernatant is then collected by centrifugation (Sorvall SS-34, 8°C at 20,000 RPM). This clarified solution is loaded onto a FPLC system equipped with an equilibrated 1 X 10 cm (QAE fast flow;
15 Pharmacia) column in 10 mM Tris HCl, pH 8.0, at a flow rate of 1 ml/min. The retained proteins are resolved with a linear NaCl gradient from 0-300 mM over 82 minutes at 3 ml/min. Fractions containing the 22 kdal apparent M.W. protein (SDS PAGE) are pooled and concentrated and buffer exchanged to 10 mM Tris Hcl, pH 9.0, by Centricon-10 (Amicon). This enriched material is further refined by HPLC
20 on a TSK DEAE 5PW (0.75 X 75 mm) column using a linear NaCl gradient (10-125 mM) in 20 mM Tris HCl, pH 9.0, over a 25 minute period. The pooled material is concentrated by Centricon 10. Other minor contaminants are finally removed by gel filtration on a TSK Bio-Sil-125 HPLC column (0.75 X 600 mm) in 50 mM phosphate, 1 M NaCl, pH 6.8. The purified recombinant protein is stored frozen at -20 C in 20
25 mM Tris HCl, pH 7.2. SDS PAGE with urea indicates the molecular weight of any of the purified recombinant MIRAP protein to be about 17,000 daltons.

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CHARTS

CHART 1

5

```
IRAP  = RPSGRKSSKMQAFRIWDVNQKTFYLRNN-QLVAGYLQGPNVNLEEKIDVVPIEPH-----
IL-1ß = APVRSLNCTL-----RDSQQKSLVMSGPYELKALHLQGQDMEQQVVFSMSFVQGEESNDK
        *    . ....      .* .**.. ...  .* * .***.... .  ...  .. .
```

10

```
IRAP  = -ALFLGIHGGKMCLSCVKSGDETRLQLEAVNITDLSENRKQDKRFAFIRSDSGPTTSFES
IL-1ß = IPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKNYP-KKKMEKRFVFNKIEINNKLEFES
        .. **... ...**** ..*.. ****.*. .. . ..* .***.* . . . . .***
```

15

```
IRAP  = AACPGWFLCTAMEADQPVSLTNMPDEGVMVTKFYFQ-EDE
IL-1ß = AQFPNWYISTSQAENMPVFLGG-TKGGQDITDFTMQFVSS
        *. *.*...*. ... ** *.. ...*  .*.* .*  ..
```

20

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CHART 2

```
      53                                              112
      ↓                                                ↓
 5    ATGGAAATCTGCAGAGGCCTCCGCAGTCACCTAATCACTCTCCTCCTCTTCCTGTTCCAT
      M   E   I   C   R   G   L   R   S   H   L   I   T   L   L   F   L   F   H   -

      113           128                              172
      ↓             ↓                                  ↓
10    TCAGAGACGATCTGCCGACCCTCTGGGAGAAAATCCAGCAAGATGCAAGCCTTCAGAATC
      S   E   T   I   C   R   P   S   G   R   K   S   S   K   M   Q   A   F   R   I   -

15    173                                            232
      ↓                                                ↓
      TGGGATGTTAACCAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGCTGGATACTTG
      W   D   V   N   Q   K   T   F   Y   L   R   N   N   Q   L   V   A   G   Y   L   -

20    233                         257                292
      ↓                           ↓                    ↓
      CAAGGACCAAATGTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCT
      Q   G   P   N   V   N   L   E   E   K   I   D   V   V   P   I   E   P   H   A   -
25

      293                                            352
      ↓                                                ↓
      CTGTTCTTGGGAATCCATGGAGGGAAGATGTGCCTGTCCTGTGTCAAGTCTGGTGATGAG
30    L   F   L   G   I   H   G   G   K   M   C   L   S   C   V   K   S   G   D   E   -

      353             370                            412
      ↓               ↓                                ↓
35    ACCAGACTCCAGCTGGAGGCAGTTAACATCACTGACCTGAGCGAGAACAGAAAGCAGGAC
      T   R   L   Q   L   E   A   V   N   I   T   D   L   S   E   N   R   K   Q   D   -

      413                                            472
      ↓                                                ↓
40    AAGCGCTTCGCCTTCATCCGCTCAGACAGTGGCCCCACCACCAGTTTTGAGTCTGCCGCC
      K   R   F   A   F   I   R   S   D   S   G   P   T   T   S   F   E   S   A   A   -

45    473                                            532
      ↓                                                ↓
      TGCCCCGGTTGGTTCCTCTGCACAGCGATGGAAGCTGACCAGCCCGTCAGCCTCACCAAT
      C   P   G   W   F   L   C   T   A   M   E   A   D   Q   P   V   S   L   T   N   -

50    533                                   584
      ↓                                      ↓
      ATGCCTGACGAAGGCGTCATGGTCACCAAATTCTACTTCCAGGAGGACGAGT
      M   P   D   E   G   V   M   V   T   K   F   Y   F   Q   E   D   E   *   -
```

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CHART 3

```
    1                                          20
    ↓                                          ↓
    M  E  I  C  R  G  L  R  S  H  L  I  T  L  L  L  F  L  F  H  -


    21             26                          40
    ↓              ↓                           ↓
    S  E  T  I  C  R  P  S  G  R  K  S  S  K  M  Q  A  F  R  I  -


    41                                         60
    ↓                                          ↓
    W  D  V  N  Q  K  T  F  Y  L  R  N  N  Q  L  V  A  G  Y  L  -


    61                            75 76 77 78 79 80
    ↓                             ↓  ↓  ↓  ↓  ↓  ↓
    Q  G  P  N  V  N  L  E  E  K  I  D  V  V  P  I  E  P  H  A  -


    81 82 83 84 85                             100
    ↓  ↓  ↓  ↓  ↓                              ↓
    L  F  L  G  I  H  G  G  K  M  C  L  S  C  V  K  S  G  D  E  -


    101            106                         120
    ↓              ↓                           ↓
    T  R  L  Q  L  E  A  V  N  I  T  D  L  S  E  N  R  K  Q  D  -


    121                                        140
    ↓                                          ↓
    K  R  F  A  F  I  R  S  D  S  G  P  T  T  S  F  E  S  A  A  -


    141                                        160
    ↓                                          ↓
    C  P  G  W  F  L  C  T  A  M  E  A  D  Q  P  V  S  L  T  N  -


    161                          177
    ↓                            ↓
    M  P  D  E  G  V  M  V  T  K  F  Y  F  Q  E  D  E  *  -
```
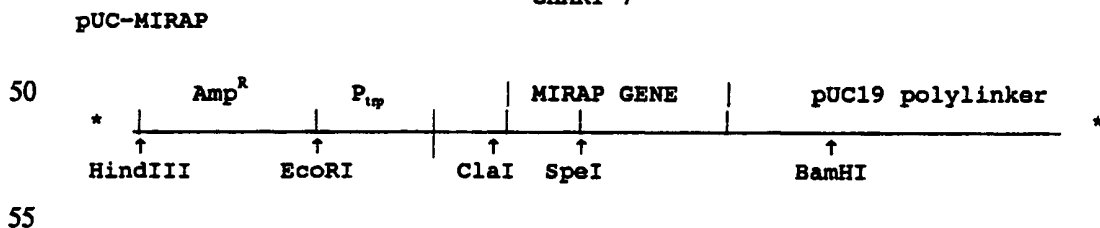
-27-

CHART 4

pTrp2-ILinh

5

| pBRori   Amp^R   P_{trp}   RBS   IRAP GENE
* |————————————|————————|————————|————————|——————↓———————|——————————————————|————— *
  ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑
Sphl   PstI   ScaI   SspI   EcoRI   ClaI   XmaI   SpeI   SspI   BamHI   BamHI

10

15

CHART 5

pUC-ILinh

20

| Amp^R   P_{trp} | IRAP GENE | pUC19 polylinker
* |————————————————|————————————————|——————|——————————|————————————————————————— *
  ↑   ↑   ↑   ↑   ↑
HindIII   EcoRI   ClaI   SpeI   BamHI

25

30

CHART 6

pTrp2-MIRAP

35

| pBRori   Amp^R   P_{trp}   RBS   MIRAP GENE
* |————————————|————————|————————|————————|——————↓———————|——————————————————|————— *
  ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑
Sphl   PstI   ScaI   SspI   EcoRI   ClaI   XmaI   SpeI   SspI   BamHI   BamHI

40

45

CHART 7

pUC-MIRAP

50

| Amp^R   P_{trp} | MIRAP GENE | pUC19 polylinker
* |————————————————|————————————————|——————|——————————|————————————————————————— *
  ↑   ↑   ↑   ↑   ↑
HindIII   EcoRI   ClaI   SpeI   BamHI

55

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CLAIMS

5
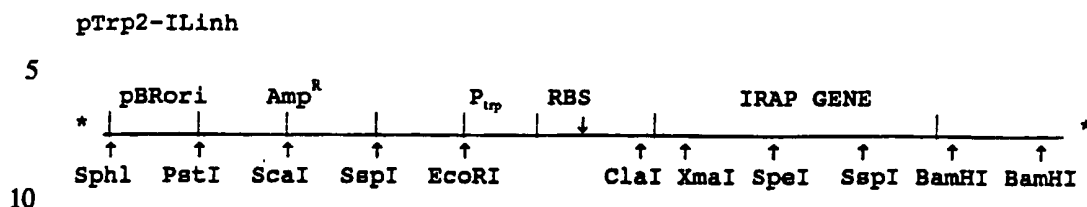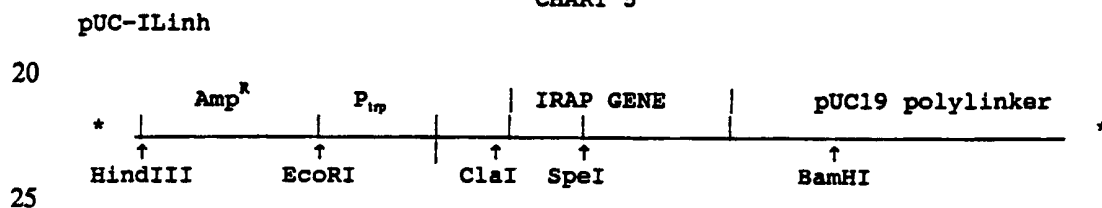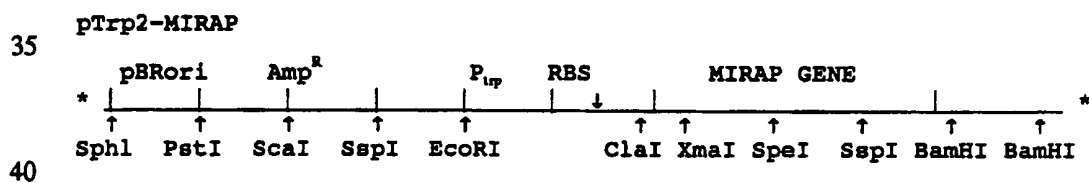
1.   A recombinant DNA molecule comprising:

(a) a nucleotide sequence encoding an IRAP protein; and,

(b) a nucleotide sequence encoding a Sclavo peptide.

2.   A recombinant DNA molecule according to Claim 1 wherein said nucleotide sequence encoding a Sclavo peptide is located within said nucleotide sequence

10 encoding an IRAP protein.

3.   A recombinant DNA molecule according to Claim 2 further comprising regulatory elements operably linked to said nucleotide sequences such that it is capable of being expressed in a suitable host.

15

4.   A recombinant DNA molecule according to Claim 2 comprising a DNA sequence which encodes an amino acid sequence selected from the group consisting of:

```
MIRAP-1              75                     85
20                    ↓                      ↓
                V Q W E E S N D K A L F L G I;

MIRAP-2              75                     85
                      ↓                      ↓
25              V Q G E E S N D K A L F L G I; and

MIRAP-3              75                     85
                      ↓                      ↓
                I E P H E S N D K I A L F L G I.
30
```

5.   A recombinant expression vector comprising a nucleotide sequence according to Claim 3.

6.   A method of preparing an improved IRAP comprising the steps of:

35 (a) culturing a suitable host transformed with an expression vector according to Claim 5 under conditions promoting expression; and

(b) purifying said improved IRAP produced thereby.

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7.   A transformed   host cell comprising a recombinant   DNA molecule according to Claim 3.

8.   Essentially   pure, improved IRAP comprising:
        a)   an IL-1 inhibitor amino acid sequence; and,
        b)   a Sclavo peptide.

9.   Essentially   pure, improved IRAP according to Claim 8 wherein said Sclavo peptide is located within said IL-1 inhibitor amino acid sequence.

10.   Essentially   pure, improved IRAP according to Claim 9 wherein said Sclavo peptide is located between amino acid residues 75 and 80 of said IL-1 inhibitor amino acid sequence.

11.   Essentially   pure, improved IRAP according to Claim 10 wherein said IRAP comprises an amino acid sequence selected from the group consisting of:

```
MIRAP-1                 75                          85
                         ↓                           ↓
                        V Q W E E S N D K A L F L G I;

MIRAP-2                 75                          85
                         ↓                           ↓
                        V Q G E E S N D K A L F L G I; and

MIRAP-3                 75                            85
                         ↓                             ↓
                        I E P H E S N D K I A L F L G I.
```


12.   A medicament   comprising essentially pure IRAP according to Claim 10.

13.   Use of a medicant according to Claim 12 for treating arthritis   in human patients.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02127

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5] : C 07 K 13/00, C 12 N 15/12, 15/62, C 12 P 21/02, C 12 N 1/21, A 61 K 37/02, //(C 12 N 1/21; C 12 R 1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	The Journal of Immunology, vol. 137, no. 10, 15 November 1986 G. Antoni et al.: "A short synthetic peptide fragment of human interleukin 1 with immunostimulatory but not inflammatory activity", pages 3201-3204, see the whole article	1-12
A	EP, A, 0343684 (SYNERGEN) 29 November 1989, see page 2; page 3; page 4, lines 1-36; claims 1,2,6-17, 25; figure 15	1-12
A	EP, A, 0231728 (SCLAVO SpA) 12 August 1987 see abstract; page 2; page 3; claims 1,2	1-12
A	WO, A, 8901946 (BIOGEN) 9 March 1989 see the claims	1-12

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19th July 1991	2 0. 09. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Mme. M. van der Drift

Form PCT/ISA/210 (second sheet) (January 1985)

	FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
A	The Journal of Immunology, vol. 143, no. 1, 1 July 1989 D. Boraschi et al.: "A monoclonal antibody to the IL-1beta peptide 163-171 blocks adjuvanticity but not pyrogenicity of IL-1beta in vivo", pages 131-134 see the whole article	1-12

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☒ Claim numbers ...13..... because they relate to subject matter not required to be searched by this Authority, namely:

PCT Rule 39.1 (iv) methods for treatment of the human or animal body by surgery of therapy as well as diagnostic methods.

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers..........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 06/09/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0343684	29-11-89	AU-A-	3746989	12-12-89
		WO-A-	8911540	30-11-89
EP-A- 0231728	12-08-87	JP-A-	62185095	13-08-87
		US-A-	4774320	27-09-88
WO-A- 8901946	09-03-89	AU-A-	2427288	31-03-89
		BE-A-	1002375	22-01-91
		EP-A-	0341273	15-11-89
		FR-A-	2621821	21-04-89
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		JP-T-	2500643	08-03-90
		NL-A-	8820676	03-07-89
		SE-A-	8901342	13-04-89

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